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(54) **Activated liposomes, method of producing them and protein coupling method using them.**

(57) Activated liposome having various material encapsulated by outer surfaces thereof are disclosed. The outer surfaces include lipid molecules, at least some of which have been modified by an oxidation reaction and which function as covalent binding sites for a variety of proteins, most particularly for IgG. When biologically active proteins are covalently bound, or coupled, to the activated liposomes, the encapsulated materials remain captured within the activated liposomes, and the coupled proteins retain a significant amount of biological activity.

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Activated Liposomes, Method of producing them and Protein Coupling method using them.

The present invention relates generally to liposomes, and more particularly to activated liposomes which encapsulate materials, such as drugs, nucleic acids, proteins and the like, and which may be readily and efficiently covalently bound to a variety of biologically active proteins without rupture of the liposomes.

Liposomes are now well recognized as useful for delivery of therapeutic agents, such as cytotoxic drugs or other macromolecules capable of modifying cell behaviour, to in vivo sites. For example, U.S. Patent 3,993,754, inventors Rahman, et al, issued November 23, 1976, discloses an improved method for chemotherapy of malignant tumors in which an antitumor drug is encapsulated within liposomes and the liposomes containing the encapsulated drug are injected into an animal or man.

It has been suggested that target, or in vivo site, specificity might be conferred on liposomes by their association with specific antibodies or lectins. Methods of associating antibodies with liposomes have been described and may be generally divided into two groups--nonspecific association and covalent attachment.

Nonspecific association appears to rely upon the affinity of the Fc portion of the antibody for the hydrophobic region of the lipid bilayer. This has little practical value because the liposomes are rendered more permeable to their encapsulated contents and may themselves be aggregated. Further, it is not known how stable this complex would be in plasma.

Previous attempts to covalently attach protein to liposomes have been unsatisfactory. For example, some of these prior attempts to covalently attach protein to liposomes have involved modifications of the proteins. This is disadvantageous because such protein modifications have tended to denature the protein, and thus a substantial loss of biological activity has ensued. Other attempts to covalently attach protein to liposomes have produced very small amounts of specific attachment, and a significant amount of encapsulated material has tended to escape during the coupling method because many of the liposomes have ruptured.

The present invention is directed to overcoming one or more of these problems.

Accordingly, it is an object of the present invention that activated liposomes be provided which may be readily and efficiently covalently bound to a variety of proteins, such as IgG, without any prior modification of the proteins.

It is another object of the present invention that such activated liposomes be highly resistant to rupture during coupling of proteins to the activated liposomes so that encapsulated contents thereof do not escape.

It is a further object of the present invention that proteins, particularly antibodies, retain a significant amount of antigen binding capacity after having been coupled to the activated liposomes.

These and other objects of the present invention are provided by activated liposomes comprising a plurality of liposomes having outer surfaces formed by modified lipid molecules. The activated liposomes have a quantity of material interior the outer surfaces thereof and encapsulated thereby. The modified lipid molecules are modified to covalently bind various protein molecules to the outer surfaces of the liposomes, which at the same time the outer surfaces retain substantially all of the material which is interior the outer surfaces and encapsulated thereby.

In other aspects of the present invention, a method for producing activated liposomes is disclosed, and a method for coupling proteins to activated liposomes is also disclosed.

The activated liposomes and method of the present invention provide that materials which are captured, or encapsulated, inside the liposomes remain captured during the activation of the liposomes and remain captured during coupling of proteins to the activated liposomes. Further, the coupled proteins retain a significant amount of biological activity. The method for coupling protein is applicable to any protein bearing primary or secondary amino groups, and is particularly useful for coupling IgG and $F[ab']_2$.

Among the end uses contemplated for activated liposomes coupled to proteins in accordance with the present inventiin is the selective targeting of various molecules to specific cell types and tissues.

Precursor, that is non-activated, liposomes useful as the starting liposomes in the present invention may be prepared by any of various conventional methods known to the art. These various known methods may be generally characterized as yielding either unilamellar vesicles or multilamellar vesicles. Either liposomal structure is suitable for the present invention. However, due to the generally larger internal space available in unilamellar liposomes, the precursor liposomes of the present invention are preferably prepared by the reverse-phase evaporation vesicle (REV) method as is described in Proc. Natl. Acad. Sci. U.S.A., Volume 75, No. 9, pp. 4194-4198 (1978), entitled "Procedure For Preparation of Liposomes With Large Internal Aqueous Space And High Capture By Reverse-Phase Evaporation", Szoka, Jr. and Papahadjopoulos, which disclosure is incorporated by herein reference.

As is known to the art, a wide variety of materials may be encapsulated by the precursor liposomes. For example, the precursor liposomes can encapsulate cytotoxic drugs, can encapsulate nucleic acids, and can encapsulate various proteins.

In any event, the precursor liposomes suitable for the present invention may be formed from either phosphatidylglycerol (hereinafter also referred to as "PG"), which has an oxidisable group at the polar head region, as the sole lipid, or may be formed from a mixture of two or more different lipids.

When formed from two or more different lipids, at least one of the lipids must contain oxidisable groups, such as vicinal amino or vicinal hydroxyl groups, along the polar head region of

the lipid molecule. For example, in the instance of vicinal amino groups, a glycolipid having galactosamine or glucosamine residue is a suitable oxidisable lipid in accordance with the present invention. More usually, at least one of the lipids will have vicinal hydroxyl groups at the polar head region. Particularly preferred as one of the lipids (that is, the oxidisable lipid) in a lipid mixture are the glycolipids such as lactosyl ceraminde, galactocerebroside, gangliosides, and trihexosyl ceramide, and the phospholipids, such as phosphatidylclycerol and phosphatidylinositol.

The amount of such lipids having oxidisable groups (generally herein referred to as "oxidisable lipids") may vary with respect to the total lipids forming the precursor liposomes; however, it is preferred that the mole percent of oxidisable lipids be in an amount of at least about 10 mole percent with respect to a total of the mixture of lipids.

Particularly preferred amounts of oxidisable lipids with respect to the total lipids are illustrated by Table I, below.

TABLE I

Oxidisable Lipid	Mole of Oxidisable Lipid To Total Lipid Mixture
Lactosylceramide	About 10
Trihexosylceramide	About 10
Galactocerebroside	About 20
Phosphatidylglycerol	About 33 - 40
Phosphatidylinositol	About 20
Gangliosides	About 10

The structures of the preferred oxidisable lipids are well known; however, for clarity Figure 1, below, illustrates PG as representative of the general

structures of the oxidisable lipids having the polar head regions and the region of non-polar tails.

Figure I: PG

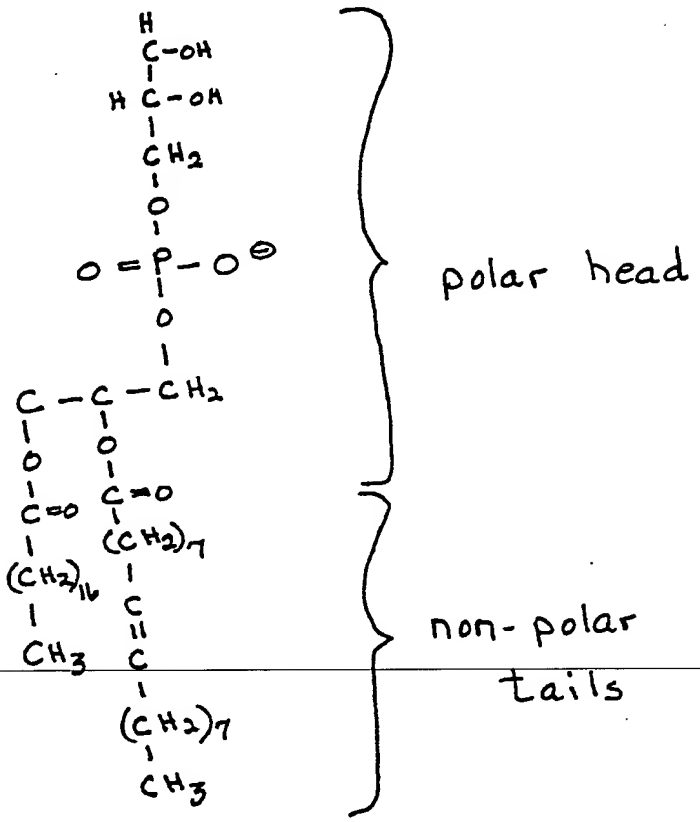


Figure 1 is generally representative of all of the lipids which may be mixed to form the precursor liposomes in defining the polar head region and the non-polar tails. The Figure 1 structure is more particularly generally representative of the oxidisable lipids which have vicinal hydroxyl groups at the polar head region thereof.

When a mixture of lipids, including the oxidisable lipid, is utilized to form the precursor liposomes, then the remaining lipid or lipids may

include any of the amphiphilic substances known to produce liposomes. A particularly preferred lipid for combination with the oxidisable lipids is phosphatidylcholine (hereinafter also referred to as "PC"), sphingomyelin or mixtures thereof.

As is known, the above discussed mixtures of lipid molecules form precursor liposomes with the lipid molecules being arranged in either one bimolecular layer (unilamellar) or a plurality of bimolecular layers (multilamellar). In any event, the most outward bimolecular layer forms an outer surface for the liposome. In an aqueous solution, the polar head regions of the lipid molecules are exposed, or extend into, the aqueous system in a generally radially outward orientation with respect to the outer surface. The non-polar tails extend radially inwardly with respect to the outer surface and form a substantially continuous hydrocarbon phase of the bimolecular layer. This substantially continuous hydrocarbon phase is relatively impermeable, and acts to encapsulate the materials inside the precursor liposomes.

Nevertheless, some mixtures of lipids forming the precursor liposomes may tend to be permeable to small molecules, and cholesterol is a desirable addition to some of these lipid mixtures for reducing the permeability of the precursor liposomes. The cholesterol tends to orientate within the bi-molecular layer. Other components may be utilized in place of cholesterol to reduce the liposome permeability. For example, a phosphatidyl choline having the fatty acid saturated aliphatic chain, or non-polar tails, of a length of 18 (rather than the usual unsaturated 16 to 18 carbon chain obtainable from egg yolks) may be

utilized. However, when sphingomyelin is mixed with the oxidisable lipid, the precursor liposomes thereof are inherently quite impermeable to small molecules.

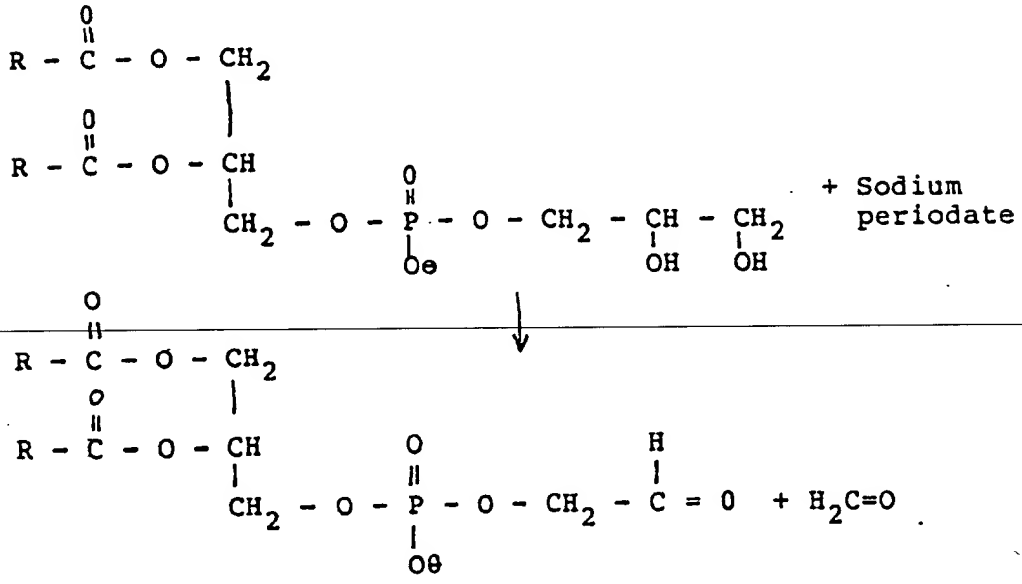
Turning to the invention, a solution of precursor liposomes is provided as has been described above. This solution is preferably a polar solution, such as an aqueous solution, but may also be a non-polar solution. The precursor liposomes are contacted with a sufficient amount of a relatively mild oxidizing reagent to produce activated liposomes. Where the lipids to be used for liposomes are in a non-polar solution, the oxidizing reagent may be lead tetraacetate. In the preferred polar solution, the oxidizing reagent of the contacting step is a periodate reagent, usually sodium periodate, which cleaves the vicinal amino or hydroxyl groups at the polar head regions of the oxidisable lipids.

Where the solution is polar and the oxidizing agent is a periodate reagent, the pH and osmolarity of the liposome solution and an added amount of periodate reagent should be substantially the same. The pH is typically about 6.0 to about 8.5. The oxidizing reagent produces activated liposomes by oxidizing the oxidisable groups, such as the vicinal hydroxyl or amino groups of the oxidisable lipid, to yield aldehyde moieties at the polar head regions of the oxidisable lipids. A sufficient quantity of periodate reagent will usually be a molar ratio with respect to the total of lipid molecules of from about 1.5:1 to about 6:1. The oxidation reaction of the contacting step is typically left to proceed for about one-half hour at room temperature, although the reaction may be permitted to proceed for up to about

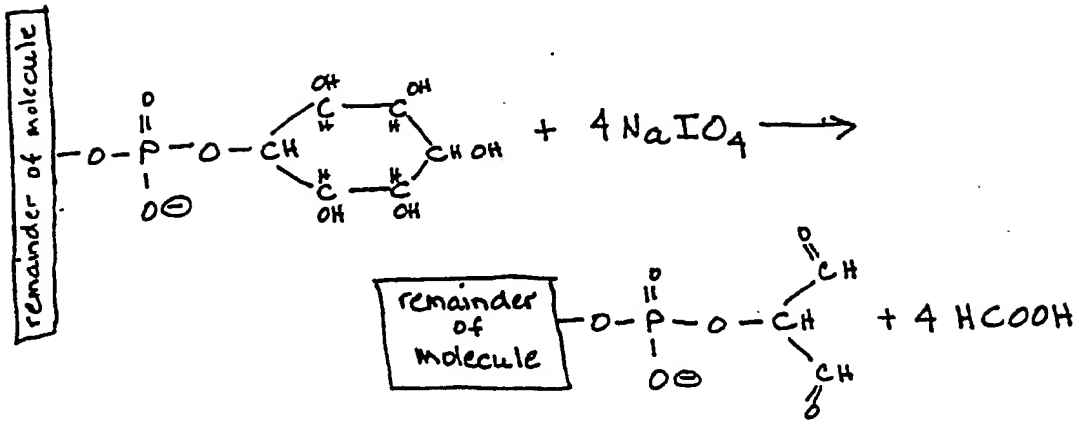
one hour on ice. The periodate reagent is then preferably removed by gel filtration through a column of dextran polymeric beads having an exclusion limit of about 75,000 daltons.

Reaction schemes I, II and III diagrammatically illustrate the activation of precursor liposomes, with the oxidisable lipids being phosphatidylglycerol, phosphatidylinositol and lactosylceramide respectively.

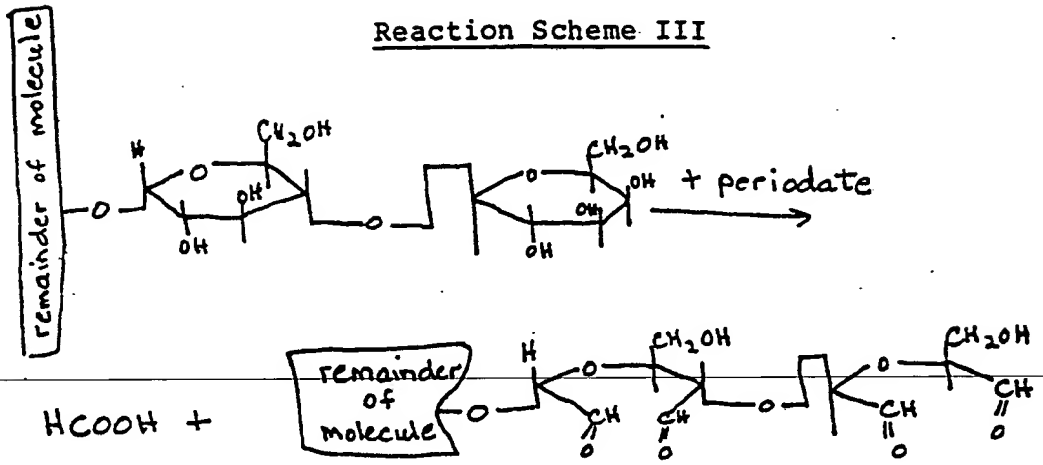
Reaction Scheme I



Reaction Scheme II



Reaction Scheme III

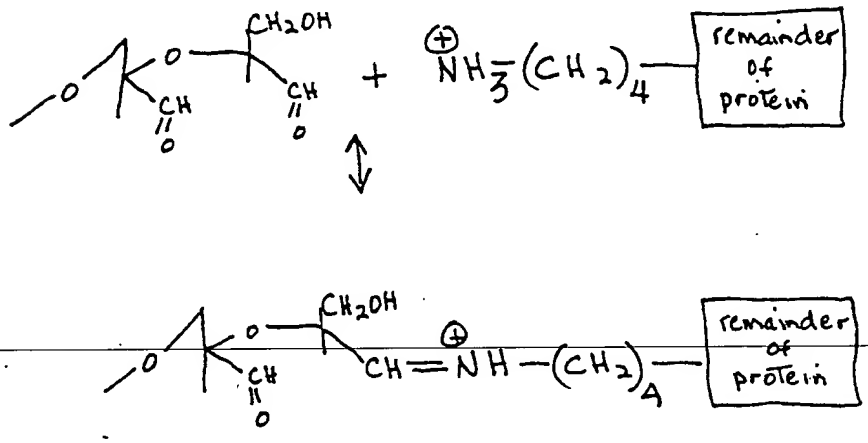


Substantially all of the material which is interior the outer surfaces of the activated liposomes remains encapsulated during the above-described oxidation with periodate reagent. As illustrated by Reaction Schemes I-III, the aldehyde moieties which are formed by the oxidation, or modification, of the oxidisable lipids at the polar head regions thereof define covalent binding sites for the protein to be bound, or coupled.

A wide variety of proteins may be attached, or coupled, to the activated liposomes. The mechanism of coupling is believed to occur between the primary or

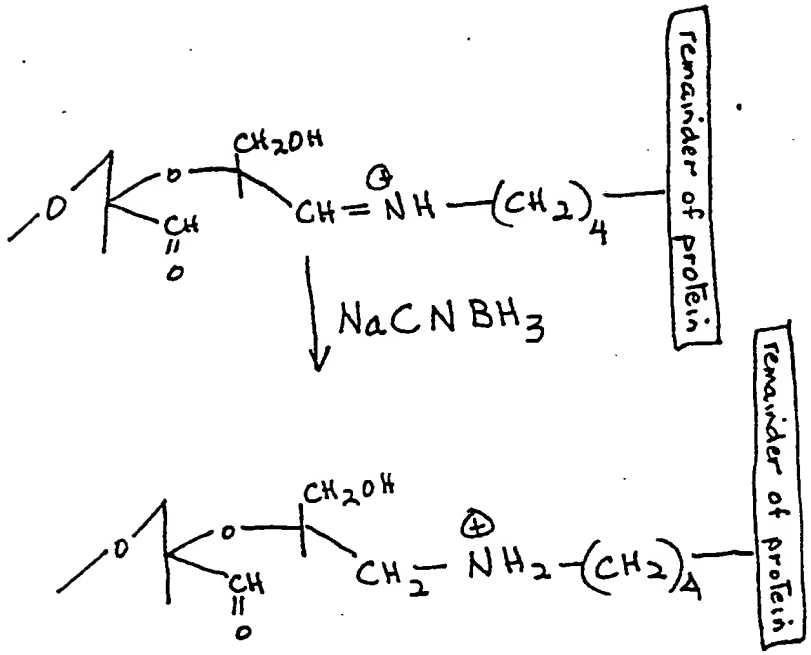
secondary amino group along the protein and the aldehyde moiety of the activated liposomes so as to form a Schiff-base, for example, with the primary amino group of a lysyl moiety. Such a mechanism is diagrammatically represented by Reaction Scheme IV, which for simplicity illustrates only the terminal galactose (after modification) of lactosylceramide.

Reaction Scheme IV



The coupling is driven to completion by a mild reducing agent, preferably sodium cyanoborohydride, so that a stable, covalent bond is formed between the protein and the activated liposome. For example, addition of a sufficient amount of sodium cyanoborohydride drives the Schiff-base of reaction scheme IV above, to completion, as is generally illustrated by Reaction Scheme V, below.

Reaction Scheme V



Reaction Schemes IV and V, above, diagrammatically illustrate coupling of a protein with lactosylceramide, where the lactosylceramide has been modified by oxidation to include aldehyde moieties. Use of the other the oxidisable lipids proceeds by an analogous manner. In the instance of modified lactosylceramide, the secondary amine moiety which is covalently binding the protein to the activated liposome may further proceed, in the presence of sodium cyanoborohydride, into an even more stable, tertiary amine form.

Although sodium cyanoborohydride is the preferred reducing agent for coupling proteins to the activated liposomes, other reducing agents may be utilized, depending upon the particular circumstances. For example, borohydride may be utilized; however, the coupling reaction would usually then be conducted at a

relatively alkaline pH, which may tend to denature the protein being coupled.

Suitable proteins for adequate coupling will have at least one primary or secondary amino group, and preferably a plurality of primary or secondary groups. Proteins having at least one and preferably at least about 20 lysyl moieties per molecule are more preferred. IgG, with about 60 lysyl moieties, has been found to be particularly well coupled; another preferred antibody for coupling with the activated liposome is $F[ab']_2$.

Four aqueous solutions of liposome, each containing from about 10 to about 40 micromoles of lipid per mililiter, were activated as previously described. The precursor liposomes had been prepared by the REV procedure and had been extruded through a polycarbonate filter to yield liposomes having a diameter of about 0.2 micron. The solutions were buffered at a pH of from about 6.0 to about 8.5. A fifth liposome solution, wherein the oxidisable lipid was not oxidized, was prepared as a control. These four activated liposome solutions in accordance with the present invention and the fifth control solution were as illustrated by Table II.

TABLE II

Solution #	Lipid Composition/ Molar Ratio	Total Lipid (micromole)	Vol.(ml)
1	PC/Lactosylceramide, 10:1	9.21	2.8
2	PC/Trihexosylceramide, 10:1	16.44	4.5
3	PC/PG, 1:1	9.5	3.1
4	Galactocerebroside/ PC/Cholesterol, 2:4:5	15	0.6
5*	PC/Lactosylceramide, 10:1	7.11	2.8

*Control solution, liposomes not activated

The five solutions as in Table II were treated as follows. 5 to 10 miligrams of IgG in the same buffer as the liposome solutions was added to the respective liposome solutions (the activated liposomes were suspended in the solutions with substantially no clumping). Sufficient sodium cyanoborohydride was added to give a concentration of about 20 milimolar, and the solutions were left for about 2 to about 3 hours at room temperature. The liposomes having covalently bound IgG thereon were then purified by conventional methods, such as column gel filtration or centrifugation. The amount of coupling is illustrated by Table III, below (the number of molecules per vesicle was estimated on the assumption that the vesicles were 0.2 micron in diameter, with about 1.8×10^{12} vesicles/micromole lipid).

TABLE III

Liposome Solution	IgG(mg) added	Protein: Lipid Ratio (g/ mole)	(g/mg lipid	Molecules IgG/vesicle
1.	20	112	147	251
2	14	57	75	128
3	14	47	62	106
4	10	96	126	216
5*	20	11	14	25

*Control solution, liposomes not activated.

IgG coupling under the above described conditions has typically resulted in the binding of from about 50 to about 200 micrograms of IgG per micromole of lipid. Substantially no coupling is observed if the liposomes have not been activated. Nonspecific binding of proteins to activated liposomes was below the limits of the protein assay utilized in determining coupling.

The proteins which may be covalently bound, or coupled, to the activated liposomes preferably exhibit bilogical activity. The coupling process in accordance with the present invention preserves a significant amount of the biological activity of the coupled protein. This is illustrated in two separate ways by use of immunopurified rabbit antifluorescein antibody, as follows.

Antifluorescein IgG binds specifically to fluorescein isothiocyanate and carboxyfluorescein. Upon binding to the antibody, the fluorescence of the fluorescein is abolished, and this was used to measure the binding activity of the antibody. Successive additions of antibody to a solution of carboxyfluorescein reduced the fluorescence due to quenching of the fluorophore upon binding to the antibody. The antigen binding capacity of liposome-bound antibody was compared by correlating the percentage reduction in fluorescence for a variety of specified protein concentrations in linear ranges where quenching was proportional to the protein concentration as illustrated by Table IV, below (wherein the original antibody, or control, linear range was from about 78/1 to about 30/4; unbound antibody was from about 85/1 to about 40/5; and coupled antibody-activated liposomes was linear over the entire range illustrated).

TABLE IV

Fluorescent Intensity/Antibody conc. x 10^8 (μmole/ml)

Original Antibody (Control)	Unbound Antibody	Coupled Antibody-Activated Liposome
78/1	85/1	95/1
60/2	75/2	90/2
40/3	62/3	85/3
30/4	50/4	78/4
18/5	40/5	70/5
12/6	35/6	65/6
8/7	30/7	60/7
8/8	20/8	58/8
	15/9	50/9

As illustrated by Table IV, above, the fluorescent quenching of carboxylfluorescein by the original antibody preparation (control) and the antibody that was recovered from the coupling process may be compared to antibody bound to the activated liposomes. If the activity of the original, control preparation is set at 100%, then the activity of the activated liposome bound antibody is about 32%, and of the recovered antibody is about 70%. Antigen binding capacity is, therefore, only partially inhibited by the inventive coupling process, and the coupled protein displays, or retains, a significant amount of antigen binding capacity.

Antibody activity of the coupled antibody was also assessed by the ability of the coupled antibody to agglutinate erythrocytes. The activated liposomes conjugated with antigen-antibody were

incubated with erythrocytes conjugated with fluorescein-isothiocyanate. This resulted in the agglutination of the erythrocytes and the haemagglutinating titre was marginally increased by the antibodies which were coupled to activated liposomes. This is illustrated by Table V, below.

TABLE V

Haemagglutination of FITC-Human Erythrocytes by Rabbit Antifluorescein IgG

Preparation	Titre (microgram/ml)
Liposomes Bound Antibody (500 molecules/vesicle)	1.22
Untreated Antibody	1.92

The titre is expressed as the minimum concentration observed to cause agglutination. Lower values indicated more effective agglutinating capacity.

The data of Table VI, below, demonstrates that the encapsulated carboxyfluorescein did not leak from PG:PC:cholesterol (1 to 1 to 1 molar ratios) REV liposomes upon activation thereof with periodate and during subsequent protein attachment procedures.

TABLE VI

Sample	Carboxyfluorescein/Lipid Ratio (mole/mole)	% of Original
Untreated Liposomes	0.260	100
Periodate Oxidized/ Desalted	0.260	100
Protein Coupled & Separated	0.286	104

Thus Table VI illustrates that the coupling does not cause the release of the encapsulated material of the activated liposomes.

In summary, the new method provides the opportunity of increasing relative uptake of material encapsulated by the coupled protein to activated liposomes by specific cells. Since the activated liposomes can be loaded with cytotoxic drugs or other macromolecules capable of modifying cell behaviour, such an increased specificity could be an important advantage for the selective targeting of various molecules to specific cell types and tissues. Such targeting could result in a substantial increase of the therapeutic index of a variety of cytotoxic drugs. In addition, such increased specificity could be used for cellular delivery of nucleic acids in gene therapy, and for delivery of proteins in enzyme replacement therapy in cases of congenital enzyme deficiencies and metabolic disorders. Possible in vivo targets for the activated liposomes may include solid tumors or circulating cells such as lymphocytes. The delivery of cytotoxic agents by the activated liposomes to lymphocytes could be of great value as a specific immunosuppressant. These and other medical applications are now within the realm of feasibility because of the existence of purified monoclonal antibodies directed against specific cells, which antibodies can be readily coupled to the activated liposomes in accordance with the present invention.

CLAIMS:

1. A method for producing activated liposomes characterised in that liposomes with outer surfaces including a plurality of lipid molecules, said lipid molecules having polar heads and non-polar tails and at least some of said polar heads with oxidisable groups thereat; are contacted with an oxidizing reagent to produce activated liposomes, the oxidisable groups being oxidized to aldehyde moieties.

2. A method according to claim 1 wherein at least some of said polar heads with oxidisable groups thereat have vicinal hydroxyl groups and are selected from the group consisting of lactosylceramide, galactocerebroside, trihexosylceramide, phosphatidylglycerol, phosphatidylinositol, gangliosides and mixtures thereof.

3. A method according to claim 2 wherein said lipid molecules are a mixture of lipids, the mixture including phosphatidylcholine or sphingomyelin.

4. A method according to claim 2 or claim 3 wherein said lipid molecules having said polar heads with vicinal hydroxyl groups are in an amount of at least about 10 molar percent with respect to a total of said mixture of lipids.

5. A method according to any one of the preceding claims wherein said lipid molecules define a bi-molecular layer and said liposomes include cholesterol disposed within said bi-molecular layer.

6. A method according to any one of claims 1 to 4

wherein said liposomes of the providing step are unilamellar vesicles having substantially equivalent diameters.

7. A method according to any one of the preceding claims wherein said oxidizing reagent is a periodate reagent or is lead tetraacetate.

8. A method according to claim 7 wherein the contacting step is conducted in a polar solvent and said oxidizing reagent is sodium periodate in a ratio with respect to the lipid molecules of from about 1.5:1 to about 6:1.

9. A method for coupling proteins to liposomes by:

activating liposomes, by a method according to any one of the preceding claims and

admixing a protein having at least one primary or secondary amino group with said activated liposomes, the admixing being conducted in a polar solvent in the presence of a sufficient quantity of sodium cyanoborohydride to bind said protein to said activated liposomes.

10. A method according to claim 9 wherein said protein has at least about 20 lysyl moieties therealong.

11. A method according to claim 9 wherein said protein is IgG or $F[ab']_2$.

12. A method according to claim 9, claim 10 or claim 11 wherein the protein is an antibody having an original, determinable antigen binding capacity prior to the admixing step, and said protein bound to said liposomes

from the admixing step has an antigen binding capacity of not less than about 10% with respect to said original, determinable antigen binding capacity.

13. Activated liposomes comprising:

a plurality of unilamellar liposomes each having a substantially predetermined diameter, said liposomes each including a substantially predetermined total quantity of lipid molecules, at least some of said lipid molecules providing a plurality of covalent binding sites on substantially each liposome, said binding sites capable of covalently binding at least about 40 micrograms of IgG per micromole of said total quantity of lipid molecules.